

ARTICLE

LRP4 Mutations Alter Wnt/ β -Catenin Signaling and Cause Limb and Kidney Malformations in Cenani-Lenz Syndrome

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Cenani-Lenz syndrome (CLS) is an autosomal-recessive congenital disorder affecting distal limb development. It is characterized mainly by syndactyly and/or oligodactyly and is now shown to be commonly associated with kidney anomalies. We used a homozygosity-mapping approach to map the CLS1 locus to chromosome 11p11.2-q13.1. By sequencing candidate genes, we identified recessive *LRP4* mutations in 12 families with CLS. *LRP4* belongs to the low-density lipoprotein (LDL) receptor-related proteins (LRPs), which are essential for various developmental processes. *LRP4* is known to antagonize *LRP6*-mediated activation of canonical Wnt signaling, a function that is lost by the identified mutations. Our findings increase the spectrum of congenital anomalies associated with abnormal lipoprotein receptor-dependent signaling.

Introduction

Spatial and temporal activation of canonical Wnt/ β -catenin signaling is an essential developmental process during organogenesis and tissue regeneration.¹ Wnt ligands bind to their specific coreceptors, such as frizzled and low-density lipoprotein-related proteins 5 and 6 (*LRP5* [MIM 603506], *LRP6* [MIM 603507]), leading to a stabilization of β -catenin and transcriptional activation.² Alteration of the *LRP5/6* signaling pathway has been described in cancer development and human diseases.^{1,3,4} *LRP4* (MIM 604270) is another member of the low-density lipoprotein receptor family, but has an antagonistic effect on *LRP5/6* signaling. Recent results of GWAS in bone mineral density⁵ and the finding that *Lrp4* serves as a receptor for sclerostin regulating bone metabolism in mice⁶ highlight the importance of *LRP4* in the regulation of bone mineral density and the development of osteoporosis. In mice, *Lrp4* dysfunction also causes syndactyly.⁷

The genetic identification of factors regulating limb formation provided important insights into the role of major signaling pathways, such as sonic-hedgehog (SHH) and fibroblast growth factor (FGF), during limb develop-

ment.^{8,9} Cenani-Lenz syndrome (CLS [MIM 212780]) is an autosomal-recessive congenital anomaly affecting mainly distal limb development. CLS is characterized by fusion and disorganization of metacarpal and phalangeal bones, radius and ulnar shortening, radioulnar synostosis, and severe syndactyly of hands and feet.^{10,11} Kidney hypoplasia has been described in one patient with CLS,¹² but is not yet regarded as an associated trait.

Here, we map the CLS1 locus to chromosome 11p11.2-q13.1 and identify mutations in the *LRP4* gene in 12 CLS families. We show that *LRP4* function is required for the physiological regulation of Wnt signaling, and we identify mutations that cause loss of *LRP4* function, which is important for normal limb and kidney development. Therefore, loss of human *LRP4* function causes syndactyly, synostosis, and renal agenesis in Cenani-Lenz syndrome.

Material and Methods

Subjects

All subjects or their legal representatives gave written informed consent for participation in the study. The study was performed in accordance to the Declaration of Helsinki protocols and

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approved by the local institutional review boards. We collected peripheral blood samples from the affected children and parents, after informed consent was obtained, according to the protocols approved by the participating institutions. All of the research procedures followed were in accordance with the ethical standards of the responsible national and institutional committees on human subject research. Fourteen families with the clinical diagnosis of CLS were included in the study. In 12 of them, mutations were identified in *LRP4*. Clinical features of some of the families have already been published; see families CL-1,¹² CL-2,¹³ CL-3,¹⁴ CL-6,¹⁵ CL-7.¹⁶ DNA from participating family members was extracted from peripheral blood lymphocytes by standard extraction procedures.

Linkage Analysis

We performed genome-wide linkage analysis in six families (CL-1 to CL-6; not all family members could be initially included into the genome scan), using the Affymetrix GeneChip Human Mapping 10K Array (version 2.0). This version of the 10K Chip Array comprises a total of 10,204 SNPs with a mean intermarker distance of 258 kb, equivalent to 0.36 cM. Genotypes were called by the GeneChip DNA Analysis Software (GDAS version 2.0, Affymetrix). We verified sample genders by counting heterozygous SNPs on the X chromosome. Relationship errors were evaluated with the help of the program Graphical Relationship Representation.¹⁷ The program PedCheck was applied to detect Mendelian errors,¹⁸ and data for SNPs with such errors were removed from the data set. Non-Mendelian errors were identified by use of the program MERLIN,¹⁹ and unlikely genotypes for related samples were deleted. Nonparametric linkage analysis using all genotypes of a chromosome simultaneously was carried out with MERLIN. Parametric linkage analysis was performed by a modified version of the program GENEHUNTER 2.1²⁰ through stepwise use of a sliding window with sets of 150 or 300 SNPs. Haplotypes were reconstructed with GENEHUNTER 2.1 and presented graphically with HaploPainter.²¹ This program also reveals informative SNP markers as points of recombination between parental haplotypes. All data handling was performed with the use of the graphical user interface ALOHOMORA,²² developed at the Berlin Gene Mapping Center to facilitate linkage analysis with chip data.

Mutation Screening

We identified candidate genes in the critical region by using the ENSEMBL and UCSC human genome databases. We amplified the 38 exons of the *LRP4* gene (primers are listed in Table S1, available online) from DNA of index patients from all 14 families and sequenced the PCR products via the BigDye Terminator method on an ABI 3100 sequencer. We resequenced all identified mutations in independent experiments, tested for cosegregation within the families, and screened at least 200 healthy control individuals from Turkey, 150 from Pakistan, 50 from Germany, and 50 from Egypt for each mutation by PCR and/or restriction digestion or direct sequencing. We analyzed all identified alterations by using the server PolyPhen. The *LRP4* protein structure was analyzed with the server Pfam in order to determine different protein domains of *LRP4*.

cDNA Analysis

RNA was extracted from fresh whole-cell blood through use of the Paxgene Blood RNA system. After cDNA transcription, nested PCR was used to amplify *LRP4* cDNA (primers are listed

in Table S1). Primers were designed according to the reference sequence.

Generation of Lrp4 Constructs

Five *Lrp4* mutant constructs were generated by site-directed mutagenesis with the use of wild-type mouse *Lrp4* in the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Karlsruhe, Germany) as template. The correct sequence of all PCR amplicons and constructs was confirmed by direct sequencing from both sides with the use of the ABI BigDye Terminator v1.1 Cycle Sequencing Kit and the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Cell Culture and Transfections

Human embryonic kidney (HEK)293T cells were cultured in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS), amphotericin B, streptomycin, and penicillin. Cells were transfected with the use of Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Luciferase Assay

One day before transfection, approx. 400,000 HEK293T cells were plated out in 12-well plates and grown up to 50% confluency in 10% FBS and DMEM. Transfections were performed in triplicate with the use of the TOP-Flash reporter system and the indicated expression plasmids with the following concentrations: 500 ng wild-type (WT) *Lrp4* or 500 ng mutants, 250 ng *Lrp6*, 250 ng *Wnt1*, 100 ng Topflash Vector, 5 ng Renilla (p-RL-TK). Cells were transfected with the use of Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Two days after transfection, cells were lysed and Luciferase activity was measured with the use of the Dual-Luciferase Reporter Assay Kit and a Glomax 96-microplate luminometer (Promega, Mannheim, Germany). Each transfection was also measured in triplicate.

Immunoblot

Immunoblot analysis was performed according to standard protocols. Detection of *LRP4* was conducted with a C-terminal *Lrp4* mouse monoclonal antibody (1:1000).

Cell-Surface Biotin-Labeling Assay

One day before the experiment, 50% confluent HEK293T cells were cotransfected with 1.5 μ g *LRP4*, WT or mutant, and 1.5 μ g insulin receptor (IR) in T75 cm² flasks. Biotinylation was carried out with the use of the Cell Surface Protein Isolation Kit (Pierce, Bonn, Germany) according to the manufacturer's instructions. Protein concentrations were measured with the use of the BCA Protein Assay Kit (Pierce). Immunoblot analysis was performed according to standard protocols. Detection of *LRP4* and IR was conducted with a C-terminal *LRP4* mouse monoclonal antibody (1:1000) and an insulin-receptor rabbit monoclonal antibody (Abcam, Berlin, Germany) (1:1000).

Results

Clinical Findings in CLS Families

We have examined 14 CLS families presenting with a variable expression of clinical symptoms. In twelve of them we identified the molecular basis of the disease (Figure 1A,

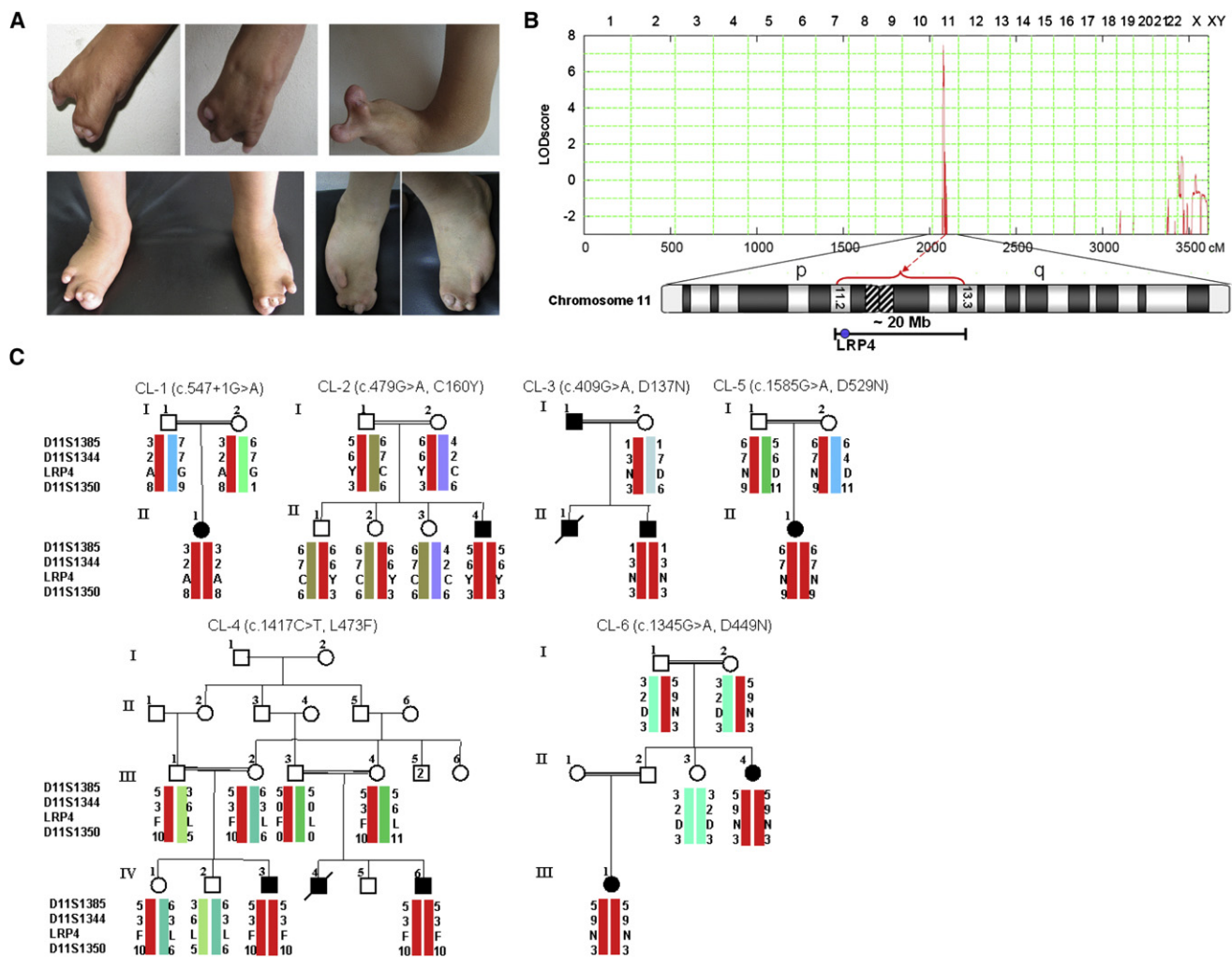


Figure 1. Clinical Findings in Families with CLS and Mapping of the *CLS1* Locus

(A) Typical hand and feet anomalies seen in CLS patients.

(B) Graphical view of additive LOD-score calculations of genome-wide SNP mapping in families CL-1 to CL-6. Ideogram of chromosome 11 showing the localization of linked region.

(C) Haplotypes of CLS families included in the initial linkage analysis.

Table 1). We observed mild facial dysmorphism in the majority of CLS cases, with prominent forehead, hypertelorism, downslanting palpebral fissures, and micrognathia. Typical limb malformations included total to partial syndactyly of hands and feet, as well as distal bone malformations affecting the radius and ulna as well as the metacarpal and phalangeal bones (Figure 1A, Table 1). Interestingly, we also found kidney anomalies, including renal agenesis and hypoplasia, in over 50% of CLS families.

Mapping of the *CLS1* Locus and Identification of *LRP4* Mutations

Initially, we genotyped DNA samples from six CLS families (CL-1 to CL-6, Figure 1) by using the Affymetrix GeneChip Human Mapping 10K Array. Affected individuals were born to consanguineous parents in all families. A combined parametric LOD score of 7.46 was obtained for a single region located on chromosome 11p11.2-q13.1

between SNPs rs1346671 and rs490192 (Figure 1B), defining a shared critical interval of about 19.7 Mb. Subsequent analysis of microsatellite markers and inclusion of additional family members confirmed homozygous haplotypes for the linked region in all affected individuals (Figure 1C). We considered *LRP4* as a highly relevant positional and functional candidate gene. No additional gene from the critical region was tested. Sequencing of the 38 coding exons of *LRP4* (Table S1) revealed different homozygous mutations in affected individuals in each of the six families. The mutations cosegregated with the disease in the families and were not found in at least 250 healthy control individuals. We found one donor splice-site mutation, c.547+1G>A (intron 6, CL-1), and five missense mutations: c.479G>A (p.C160Y, exon 5, CL-2), c.409G>A (p.D137N, exon 4, CL-3), c.1417C>T (p.L473F, exon 12, CL-4), c.1585G>A (p.D529N, exon 13, CL-5), c.1345G>A (p.D449N, exon 12, CL-6) (Figure 2A). All missense

mutations are located in the extracellular domains of LRP4 within highly conserved regions, as shown by LRP4 protein alignments of various species (Figure S1). p.C160Y and p.D137N mutations lie within the ligand-binding (class A) repeat-containing domain of the receptor, p.D449N and p.L473F are located within calcium-binding epidermal growth factor (EGF) repeats, and D529 is located within the YWTD domain (Figure 2C).

We continued the molecular analysis of *LRP4* in eight additional CLS families (Figures 3A and 3C). In families CL-9 and CL-10, we found the same homozygous p.D529N mutation as identified before in the CL-5 family. All three families originated from Turkey, and haplotype analysis confirmed that p.D529N is a common founder mutation in Turkish CLS patients (Figure 3C). It is also interesting to note that the p.D137N mutation is repeatedly found in CLS families from Egypt, as we could identify a second family, CL-12, carrying this mutation. p.D137N in both families was located on identical haplotypes (Figure 3D), suggesting that p.D137N is a founder mutation. Two additional missense mutations were found: c.1382A>C (p.T461P, exon 11, CL-7) in a Jordanian patient and c.3049T>C (p.C1017R, exon 22, CL-8) in a large CLS family from Pakistan with six affected family members (Figure 2B, Figure 3A). These mutations also cosegregated with the disease, were not found in matched controls, and were located in highly conserved regions (Figure S1). Furthermore, no *LRP4* mutation was found in two other consanguineous CLS families, and haplotype analysis did not show homozygosity of the *LRP4* region in affected individuals from both families, supporting the idea of further locus heterogeneity (Figure 3B).

We found initial evidence for an impairment of LRP4 function as the underlying pathomechanism of CLS by identifying compound-heterozygous splice-site mutations in a typically affected fetus with CLS. Both mutations, c.200-9G>A and c.4959G>C (Figure 2C), caused aberrantly spliced *LRP4* transcripts (r.199_200insGATTCAG and r.4952_4987del, respectively), and both mutations lead to a truncated protein (Figures 4A and 4B).

LRP4 Mutations Cause Loss of Protein Function

To investigate whether the identified missense mutations confer loss of function or whether they are functionally hypomorphic with biochemically detectable residual protein activity, we analyzed the effect of five missense mutations (p.D137N, p.C160Y, p.L473F, p.D449N, and p.D529N) on the transduction and activation of canonical Wnt signaling by using a Dual Luciferase Reporter Assay in transiently transfected HEK293T cells. Consistent with earlier findings,⁵ we found that WNT1 was able to significantly activate LRP6-mediated β -catenin signaling and that additional coexpression of LRP4 potently antagonized this activation (Figure 5A). In contrast, coexpression of each of the five missense mutations abolished the observed antagonistic LRP4 effect on LRP6-mediated activation of Wnt/ β -catenin signaling. Moreover, mutant

LRP4 receptors failed to be efficiently transported to the plasma membrane, as shown by cell-surface biotinylation (Figure 5B).

Discussion

We report the mapping of the CLS1 locus to chromosome 11p11.2-q13.1 and present convincing evidence that mutations in the *LRP4* gene cause CLS. Clinical findings in our CLS patients showed that in addition to the well-described distal limb malformations (ranging from total to partial syndactyly and bone malformations of both hands and feet), patients presented with facial features such as prominent forehead, hypertelorism, downslanting palpebral fissures, and micrognathia. Previously, renal hypoplasia has been reported in only one case¹⁶ and was therefore not regarded as an associated trait of CLS. Our finding that over 50% of CLS families present with renal agenesis and/or hypoplasia adds kidney anomalies to the clinical spectrum of CLS. In this context, it is interesting to note that a subpenetrant phenotype of kidney agenesis was observed in *Lrp4* homozygous null mice. In the *Lrp4*^{-/-} homozygous kidneys the ureteric budding is often delayed, resulting in insufficient stimulation of the mesenchyme. This results in destruction of prenephric mesenchymal structures, and no kidneys are formed (J. Herz, personal communication). These findings clearly show that *Lrp4* has an important function for kidney development in mice and humans.

Recently, murine LRP4 was shown to serve as a coreceptor for agrin in the formation of the neuromuscular junction.²³ Mutations in genes encoding other members of this complex have been associated with akinetic and myasthenic syndromes in humans (MIM 288150 and MIM 254300).^{24,25} Given that we did not observe a clinically detectable neuromuscular phenotype in our CLS patients, the role of LRP4 in the development and function of the neuromuscular junction in humans (as opposed to mice) will require further investigation. In the case that the *LRP4* mutations found in CLS patients are not complete loss-of-function mutations and do have minor residual functionality in vivo, there could be a different but overlapping phenotype caused by, for example, homozygous nonsense mutations or deletions in *LRP4*, and this could include neuromuscular symptoms.

The *LRP4* mutations identified in our study are frequently missense mutations, which are located in the large extracellular domain of LRP4 within the ligand-binding (class A) repeat-containing domain, calcium-binding EGF repeats, and the YWTD domain of the receptor (Figure 2C). These changes might cause structural alterations of the extracellular LRP4 domain that interfere with normal folding and thus prevent the efficient export of the protein through the secretory pathway, but this hypothesis has to be proven in future experimental studies. Our functional analysis of five of the missense mutations clearly

Table 1. Clinical Findings in CLS Families Carrying *LRP4* Mutations

[illegible]

Table 1. Continued

Family Data	CL-1	CL-2	CL-3	CL-4	CL-5	CL-6	CL-7	CL-8	CL-9	CL-10	CL-11	CL-12
Disorganized / missing metatarsals and phalanges	-	-	+	+	+/-	-	+	+	+	-	-/+	+
Nail aplasia /partial	-	-	+	+	+/-	+	+	+	-/+	-	+	+
Kidney anomalies												
Agenesis	-	-	-	-	-	-	unilateral	-	bilateral	bilateral	bilateral	-
Hypoplasia	bilateral	-	-	-	-	unilateral	-	unilateral	-	-	-	-
Ectopic localization	-	-	-	-	-	+	-	+	-	-	-	-
Additional findings												
Developmental delay	-	-	+	-	-	-	-	-	-	mild gross motor delay	?	?
Other		bilateral broad hallux valgus anomaly		hypoplastic scrotum	scoliosis, hemivertebrae, mixed-type hearing loss	duplicated distal phalanges of the first and second toe, congenital cataract	pulmonary stenosis, congenital hip dislocation	hypothyroidism	G1: died at first day of life due to bilateral renal agenesis	G2: medical abortion (20. GW), bilateral renal agenesis	medical abortion (20. GW)	pectus excavatum

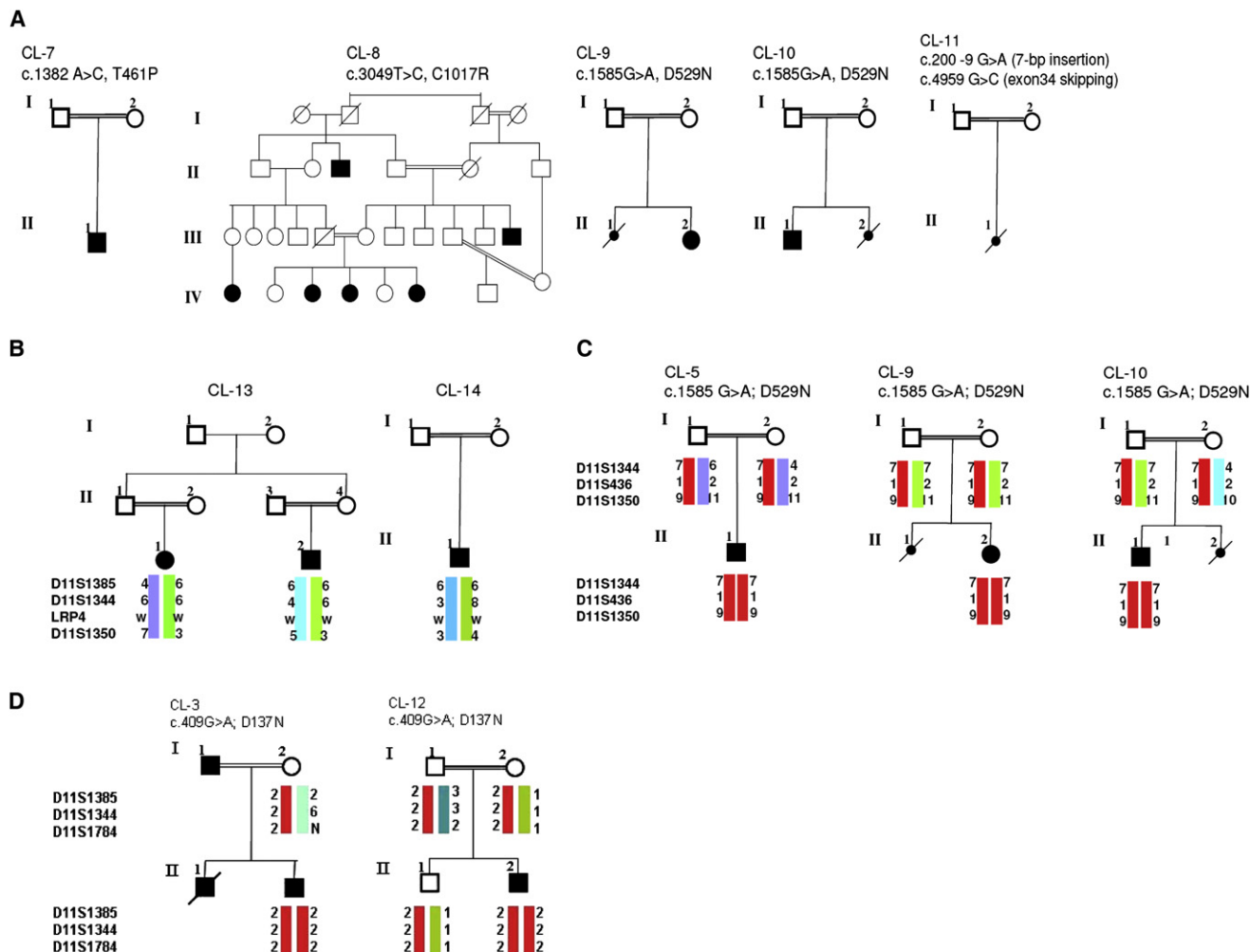


Figure 3. Additional CLS Families and Genetic Heterogeneity

(A) Pedigrees of additional CLS families.

(B) The *LRP4* locus was excluded in families CL-13 and CL-14 by haplotype analysis.

(C and D) Identification of an *LRP4* founder mutation, p.D529N, in three Turkish CLS families (C) and of p.D137N in two families from Egypt (D). Identical haplotypes are shown in red.

altered Wnt signaling. Future approaches are needed to show that knocking down *LRP4* expression upregulates Wnt/ β -catenin in vivo.

In two CLS families, we did not find *LRP4* mutations, and haplotype analysis did now show homozygosity in affected individuals born to consanguineous parents. In the CL-13 family with only a single affected individual, homozygosity is only an assumption due to parental consanguinity, but lack of homozygosity does not completely exclude *LRP4* as causative gene. In the CL-14 family, haplotype analysis of microsatellite markers as well as results from 250K array analysis excluded common haplotypes in both affected individuals, suggesting further locus heterogeneity in CLS. Future identification of a causative gene(s) in these families will highlight additional key proteins for distal limb development.

We conclude that *LRP4* function is required for the physiological regulation of Wnt signaling, which is important for normal limb and kidney development. Homozygous

loss of human *LRP4* function causes syndactyly, synostosis, and renal agenesis in Cenani-Lenz syndrome.

Supplemental Data

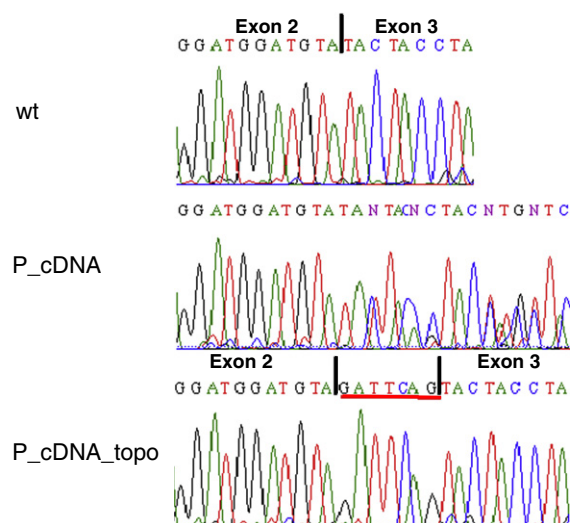
Supplemental Data include one figure and one table and can be found with this article online at <http://www.ajhg.org>.

Acknowledgments

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A

CL-11: c.200-9G>A
c.199_200insGATTGAG; p.I67RfsX77



B

CL-11: c.4959G>C
c. 4952_4987del; p.V1651DfsX1691

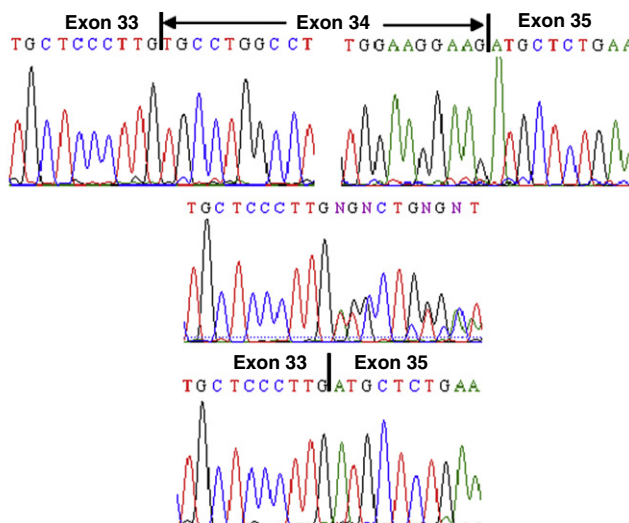


Figure 4. Splicing Effects of Mutations in Family CL-11

(A) Analysis of the heterozygous c.200-9G>A mutation. cDNA sequences of the exon 2-exon 3 boundary of wild-type (wt) and patient cDNA are shown (without [P_cDNA] and after subcloning via the TOPO-Vector system [P_cDNA_topo]).

(B) Analysis of the heterozygous c.4959G>C mutation. Electropherograms show the cDNA sequences of *LRP4* transcripts encoded by exon 33 to exon 35 of wild-type (wt), and skipping of exon 34 in the patient cDNA.

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Web Resources

The URLs for data presented herein are as follows:

ENSEMBL, <http://www.ensembl.org>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim/>

Pfam Server, <http://pfam.sanger.ac.uk/>

PolyPhen, <http://coot.embl.de/PolyPhen>

UCSC Genome Browser, <http://www.genome.ucsc.edu>

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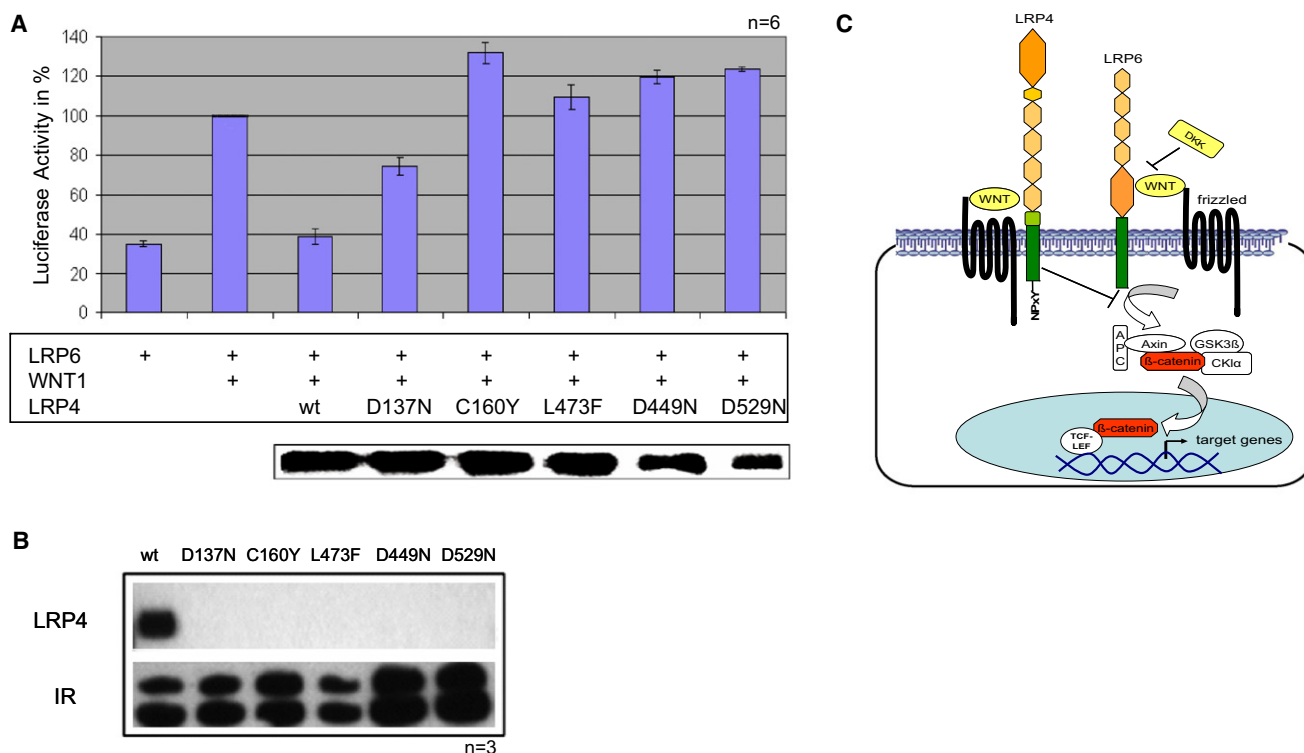


Figure 5. Functional Analysis of LRP4 Missense Mutations

(A) Results from the Dual Luciferase Reporter Assay after coexpression of LRP6, WNT1, and wild-type and mutant LRP4 in different combinations. Graph depicting the relative Luciferase activities (mean and standard deviations of six experiments, in triplicate each time). Comparable steady-state expression levels of wild-type and mutant LRP4 proteins are shown by immunoblotting of total lysates after measurement of luciferase activity.

(B) Compared to wild-type LRP4 and wild-type insulin receptor (IR), mutant LRP4 proteins are not detectable by cell-surface biotinylation. Results from three independent experiments are shown.

(C) Schematic representation of LRP4 function in LRP6-mediated activation of Wnt/β-catenin signaling.

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